REMARKS

1. Status of claims

After entry of the above amendment, claims 1-7 and 10 are pending.

2. Support for amendment

The above amendment finds support in the specification at p. 9, line 17 of the clean copy of the substitute specification, filed January 15, 2004, and in the state of the art known to the skilled artisan as of the priority date of the present specification, indicated by http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=pubmed&dopt=Abstract&list_uids=9813245, abstract of Magrath et al., Gene. (1998 Nov 5);222(1):69-75; http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&list_uids=9483796&dopt=Abstract, abstract of Van Rensburg et al., Yeast. (1998 Jan 15);14(1):67-76; http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=pubmed&dopt=Abstract&list_uids=9707436, abstract of Greger et al., EMBO J. (1998 Aug 17);17(16):4771-9; all accessed by the undersigned on October 21, 2004, and Baldari *et al.* EMBO Journal, vol. 6, no. 1, pp. 229-234 (1987). For the Examiner's convenience, copies of the documents referred to above are attached hereto. No new matter has been added by this amendment.

3. Claim objections

The Examiner objected to claim 2 for containing the undefined abbreviation ARS, and to claim 7 for containing the undefined abbreviations ADH1, GAL, and CYC1. Claim 2 now recites a definition for the abbreviation ARS, and claim 7 recites definitions for the abbreviations

ADH1, GAL, and CYC1. In light of the above amendment, Applicants submit the basis for this objection to claims 2 and 7 has been removed.

4. Claim rejections under 35 U.S.C. §112

The Examiner rejected claim 10 under 35 U.S.C. §112, second paragraph, as being indefinite. Specifically, the Examiner found the phrase "and/or nucleic acids" to be unclear on the grounds that a recitation of transformation in the absence of nucleic acids is unclear.

In light of the above amendment, Applicants point to the use of one or more nucleic acids as a carrier in a transformation process, discussed at p. 9, line 17 of the specification. Applicants submit claim 10, as amended, is clear, and request this rejection be withdrawn.

The Examiner also rejected claims 1-7 under 35 U.S.C. §112, first paragraph, as containing new matter, specifically, the recitation of "recovering the protein" in claim 1.

The specification discusses the "recovery" of gene products, i.e., proteins, at p. 2, lines 13-15 and p. 8, lines 4-7 and 12-15. The specification reports the collection of a β-galactosidase fraction and its assay at p. 13, lines 13-21. The specification discusses the production of gene products throughout, such as the Background of the Invention section, in which various uses of gene products are reported. It is clear to the skilled artisan that "production" in this context includes the recovery of gene products. Therefore, Applicants submit claim 1 does not contain new matter, and request this rejection be withdrawn.

5. Claim rejections under 35 U.S.C. §102

The Examiner rejected claims 1, 5, and 10 under 35 U.S.C. §102(e) as being anticipated by Gilbert et al., U.S. 2002/0034805 ("Gilbert"). Applicants respectfully traverse this rejection for the reasons set forth below.

Attached hereto is a Declaration under 37 CFR 1.131 of Danilo Porro, an inventor of the present application (the "Porro Declaration"). As the Porro Declaration makes clear, the invention as presently claimed was reduced to practice between January 1, 1996 and September 11, 1998. Gilbert was filed on December 14, 1998. Therefore, the present invention was made before the effective date of Gilbert, and Applicants submit this rejection of claims 1, 5, and 10 should be withdrawn.

The Examiner also rejected claims 1 and 5 under 35 U.S.C. §102(b) as being anticipated by Sjoberg, US 6,500,661, ("Sjoberg"). Applicants traverse this rejection in light of the claimed priority of the present application.

Sjoberg issued on December 31, 2002, based on an application filed January 14, 1999. Applicants point to their priority claim to prior copending application 10/130,061, filed May 15, 2002, which was a national phase entry of PCT/EP00/00268, filed January 14, 2000, which claimed priority from Italian application MI99A000065, filed January 15, 1999. Applicants' earliest priority date, January 15, 1999, predates the date of issuance of Sjoberg, and thus they submit Sjoberg cannot be prior art under 35 U.S.C. §102(b) and this rejection should be withdrawn.

If the Examiner is minded to reject any of the present claims under Sjoberg under a different paragraph of 35 U.S.C. §102, Applicants wish to direct her attention to the Porro Declaration, discussed above. The invention as presently claimed was reduced to practice

between January 1, 1996 and September 11, 1998, prior to the filing date of Sjoberg, January 14, 1999.

6. Conclusion

Applicants submit all pending claims 1-7 and 10 are in condition for allowance. The Examiner is invited to contact the undersigned patent agent at (713) 934-4065 with any questions, comments or suggestions relating to the referenced patent application.

Respectfully submitted,

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CUSTOMER NO. 23720

December 21, 2004

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AGENT FOR APPLICANTS

1: Gene. 1998 Nov 5;222(1):69-75. Related Article

ELSEVIER SCIENCE
FULL-TEXT ARTICLE

Overlapping 3'-end formation signals and ARS elements: tightly linked but functionally separable.

Magrath C, Lund K, Miller CA, Hyman LE.

Tulane University, Interdisciplinary Program in Molecular and Cell Biology, Orleans, LA, USA.

3'-End formation signals are closely associated with autonomous replicating sequences (ARSs) in Saccharomyces cerevisiae in that ARSs frequently cont signals that direct 3'-end formation (Chen et al., 1996). Mutationally-inactiva ARSs that co-reside with 3'-end formation sequences do not disrupt 3'-end formation, thus demonstrating that replication function does not affect termir function. To test the corollary possibility that 3'-end formation is important f replication function, we made point mutations in ARS305 that increase readt of the 3'-end formation signals and determined plasmid replication efficiency Replication efficiency, as assessed by plasmid stability assays, was not altere mutations affecting 3'-end formation when transcription through the ARS wa either absent or highly-induced. Under conditions of high-level transcription through the ARS, the rate of plasmid loss in both wild-type and mutated terminators increased over five-fold from rates observed during transcription repressed conditions. This result indicates that the native 3'-end formation sig incapable of protecting the replication function when high levels of transcripi are directed into the ARS. Thus, the compact nature of the S. cerevisiae geno rather than a functional inter-dependence, may account for close association transcription terminators and ARSs.

PMID: 9813245 [PubMed - indexed for MEDLINE]

Oct 13 2004 06:44:09

1: Yeast. 1998 Jan 15;14(1):67-76.

Engineering yeast for efficient cellulose degradation.

Van Rensburg P, Van Zyl WH, Pretorius IS.

Institute for Wine Biotechnology, University of Stellenbosch, South Africa.

Saccharomyces cerevisiae produces several beta-1,3-glucanases, but lacks th multicomponent cellulase complexes that hydrolyse the beta-1,4-linked gluc polymers present in cellulose-rich biomass as well as in haze-forming glucan certain wines and beers. We have introduced into S. cerevisiae a functional cellulase complex for efficient cellulose degradation by cloning the Endomy fibuliger cellobiase (BGL1) gene and co-expressing it with the Butyrivibrio fibrisolvens endo-beta-1,4-glucanase (END1), the Phanerochaete chrysospor cellobiohydrolase (CBH1) and the Ruminococcus flavefacies cellodextrinase (CEL1) gene constructs in this yeast. The END1, CBH1 and CEL1 genes we inserted into yeast expression/secretion cassettes. Expression of END1, CBH CEL1 was directed by the promoter sequences derived from the alcohol dehydrogenase II (ADH2), the phosphoglycerate kinase I (PKG1) and the alc dehydrogenase I (ADH1) genes, respectively. In contrast, BGL1 was express under the control of its native promoter. Secretion of End1p and Cel1p was d by the signal sequence of the yeast mating pheromone alpha-factor (MF alph whereas Cbh1p and Bgl1p were secreted using their authentic leader peptide construction of a fur1 ura3 S. cerevisiae strain allowed for the autoselection c multicopy URA3-based plasmid in rich medium. S. cerevisiae transformants secreting biologically active endo-beta-1,4-glucanase, cellobiohydrolase, cellodextrinase and cellobiase were able to degrade various substrates includ carboxymethylcellulose, hydroxyethylcellulose, laminarin, barley glucan, cellobiose, polypectate, birchwood xylan and methyl-beta-D-glucopyranosid study could lead to the development of industrial strains of S. cerevisiae capa converting cellulose in a one-step process into commercially important commodities.

PMID: 9483796 [PubMed - indexed for MEDLINE]

Oct 13 2004 06:44:09

1: EMBO J. 1998 Aug 17;17(16):4771-9.

Related Article

nature publishing group

Poly(A) signals control both transcriptional termination and initiation between the tandem GAL10 and GAL7 genes of Saccharomyces cerevisiae.

Greger IH, Proudfoot NJ.

Sir William Dunn School of Pathology, University of Oxford, South Parks R Oxford OX1 3RE, UK.

We have investigated transcriptional interactions between the GAL10 and G genes of Saccharomyces cerevisiae. Both genes are part of the galactose (GA gene cluster which is transcriptionally activated to high levels in the presenc galactose. Since GAL7 is positioned downstream of GAL10 and both genes a expressed co-ordinately at high levels, the possibility that GAL10 transcripti influences GAL7 was analysed. Using transcriptional run-on assays, we shov high levels of polymerase are found in the 600 bp GAL10-7 intergenic regio accumulate over the GAL7 promoter. Furthermore, GAL7 transcription is enhanced when the GAL10 upstream activating sequence (UASG) is deleted indicating that interference between GAL10 and GAL7 is likely to occur in t chromosomal locus. Deletions in the GAL10 poly(A) signal result in comple inactivation of the GAL7 promoter and cause a dramatic increase in bi-cistro GAL10-7 mRNA, predominantly utilizing the downstream, GAL7 poly(A) s These data demonstrate a pivotal role for the GAL10 poly(A) site in allowing simultaneous expression of GAL10 and GAL7. In effect, this RNA processin signal has a direct influence on both transcriptional termination and initiation

PMID: 9707436 [PubMed - indexed for MEDLINE]

The EMBO Journal vol.6 no.1 pp.229-234, 1987

A novel leader peptide which allows efficient secretion of a fragment of human interleukin 1β in *Saccharomyces cerevisiae*

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Communicated by M.L.Melli

Killer strains of *Kluyveromyces lactis* secrete a toxin which presumably is processed during secretion from a larger precursor. Analysis of the sequence of the *K. lactis* killer toxin gene predicts that the first 16 amino acids at the amino terminus of the protein should represent its leader peptide. We have tested the capability of this leader peptide to direct secretion of a protein fused to it by inserting a synthetic oligonucleotide identical to the sequence of the putative leader peptide into a yeast expression vector. Subsequently, the cDNA coding for the secreted active portion of the human interleukin 1β (IL-1β) was fused to the leader peptide sequence of the killer toxin. This construction in *Saccharomyces cerevisiae* is capable of directing synthesis and secretion of correctly processed IL-1β into the culture medium.

Key words: expression vector/signal peptide/killer toxin/yeast/interleukin 1β

Introduction

The leader peptide sequences of the α mating pheromone (α-factor) and the killer toxin of *Saccharomyces cerevisiae* have been extensively used in recent years to construct vectors for secretion of homologous and heterologous gene products in yeast. The efficiency of secretion directed by such vectors varies considerably, ranging from complete secretion as in the case of EGF (Brake *et al.*, 1984) to only partial secretion of the synthesized protein as in the case of human IFN-α1 (Singh *et al.*, 1984) or murine IL-2 (Miyajima *et al.*, 1985). Both the α-factor and the killer toxin of *S. cerevisiae* have a long leader peptide which requires special processing and is quite different from the typical leader peptides of multicellular organisms (Julius *et al.*, 1984; Tipper and Bostian, 1984).

Studies on the yeast *Kluyveromyces lactis* have shown that killer strains of this species secrete a toxin that is structurally and functionally different from the *S. cerevisiae* killer toxin (Sugisaki *et al.*, 1983). The *K. lactis* killer toxin is composed of two subunits and is released into the culture medium as a glycoprotein (Sugisaki *et al.*, 1984). The presumptive leader peptide deduced from the nucleotide sequence of the *K. lactis* toxin gene (Stark *et al.*, 1984) should be only 16 amino acids long with a structure similar to that of both prokaryotic and eukaryotic leader peptides (Perlman and Halvorson, 1983). It seemed interesting to see whether this sequence, placed in front of a heterologous cDNA in a yeast expression vector, could direct efficient secretion of the protein into the culture medium. For this purpose, we have constructed a yeast vector containing an inducible hybrid promoter upstream of the putative *K. lactis* toxin leader peptide sequence, as well as elements for selection and episomal replication in *S. cerevisiae*.

The cDNA coding for the portion of human interleukin 1β corresponding to its secreted active form (March *et al.*, 1985), minus the first four *N*-terminal amino acids, was fused in frame to the leader peptide sequence of the killer toxin. This construction in *S. cerevisiae* is capable of directing synthesis and complete secretion of correctly processed and active interleukin 1β (IL-1β) into the culture medium.

Results

Construction of YEpsec1

The secretion vector YEpsec1 was derived from the yeast expression vector pEMBLyex2, as illustrated in Figure 1A,B. pEMBLyex2 contains two blocks of yeast elements in addition to bacterial sequences necessary for selection and replication of the plasmid in *Escherichia coli*. The first, which determines episomal replication and copy number, is derived from plasmid pJBD219 (Beggs, 1978). It comprises a 3220-bp *Nde*I – *Sal*I fragment spanning the *leu2-d*, 2μm *STB* and *ORI* portions of pJBD219. This fragment also includes a small part of the 3' end of the *FLP* gene of the 2-μm plasmid, which provides transcription termination and polyadenylation signals to sequences cloned in the polylinker, ~205 bp downstream of the polylinker *Hin*dIII site (Sutton and Broach, 1985). The second is the *Hin*dIII – *Bam*HI fragment from plasmid G2 (Guarente, 1983) that carries the *URA3* gene and signals which induce transcription into the polylinker during growth on galactose as a carbon source. These transcription signals derive from a hybrid promoter of a fusion between the GAL 'upstream activation sequence' (UAS_G) and the 5' non-translated leader of the yeast *CYC1* gene, up to position −4 from the ATG translation initiation codon. Translation starts at the first ATG of a fragment inserted in the polylinker.

The last step of the construction was to insert a synthetic oligonucleotide between the *Sst*I and *Kpn*I sites of pEMBLyex2 to give YEpsec1.

The synthetic oligonucleotide, shown in Figure 1B, corresponds to the putative leader peptide sequence of the *K. lactis* killer toxin gene (Stark *et al.*, 1984), and it comprises the sequence coding for the aminoterminal 16 amino acids of the killer toxin from the initiator methionine to the presumptive signal peptidase cleavage site Val-Gln-Gly. Thus, the vector YEpsec1 has an element for directing secretion, positioned between an inducible hybrid promoter (GAL–CYC) and the transcription termination signals (3' end of *FLP*).

The ability of YEpsec1 to direct expression and secretion of heterologous gene products was tested. This was done by cloning a cDNA coding for amino acids 12.1 – 269 of human IL-1β (Auron *et al.*, 1984) into the *Bam*HI site situated immediately downstream of the killer toxin leader peptide sequence of YEpsec1. The mature secreted form of human IL-1β consists

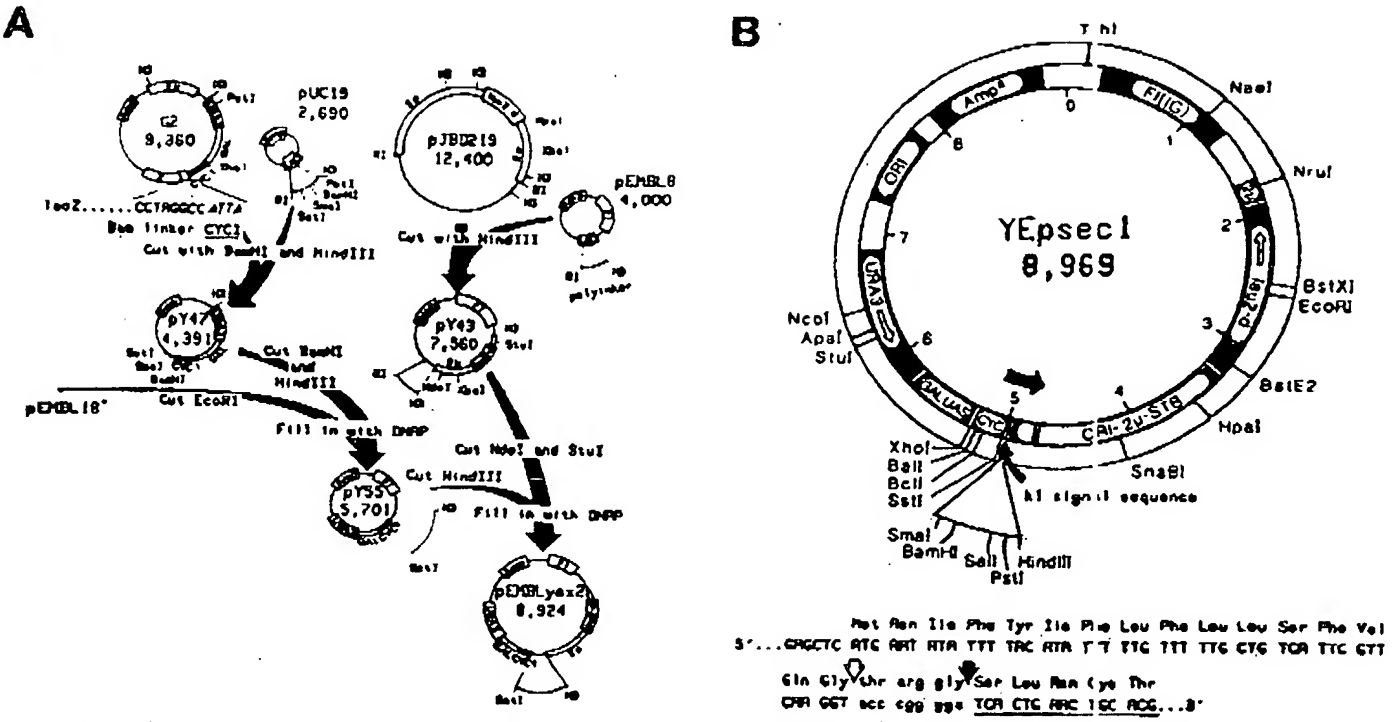

Fig. 1. Schematic presentation of plasmid constructions. Panel A Plasmid pY47 was constructed by inserting the 1700-bp *Bam*HI–*Hin*dIII fragment of plasmid G2 (Guarente, 1983) into pUC19. The *Pst*I site in the *URA3* gene was removed by treatment of *Pst*I digested DNA with DNA polymerase I, Klenow fragment (DNAP). This same fragment was excised, rendered blunt-end with DNAP and inserted in the filled *Eco*RI site of pEMBL 18* (Dente *et al.*, 1985). To construct pY43 the 3560-bp *Hin*dIII fragment from pJBD219 (Beggs, 1978) carrying *leu2-d*, and from 2-μm *STB*, *ORI*, one complete inverted repeat and the 3' end of the *FLP* gene was inserted into pEMBL 8. A smaller fragment with the same features was excised from pY43 with *Nde*I and *Stu*I and inserted blunt-end into the *Hin*dIII site of pY55 to create pEMBLyex2. The abutting filled *Hin*dIII–*Nde*I sites recreate the *Hin*dIII restriction site. Panel B Map of the secretion vector YEpsec1 showing unique restriction sites. The large arrow indicates the direction of transcription and the double line the termination and polyadenylation signal from the *FLP* gene. The sequence of the *K. lactis* killer toxin leader peptide (K1 signal sequence) and of the junction between the signal sequence and the IL-1β cDNA cloned in YEpsec1 is reported in the lower part of the panel. The hypothetical cleavage site for the killer toxin leader peptide is shown by an empty arrow, whereas the filled arrow indicates the cleavage site determined experimentally. The part of the sequence given in lower case represents the polylinker sequence at the junction and the sequence from IL-1β is underlined.

of amino acids 117–269 of the precursor molecule (March *et al.*, 1985), therefore the cDNA cloned in Yepsec1 codes for a fragment of IL-1β which represents the complete active form of the molecule except for the absence of the first four *N*-terminal amino acids. It should also be noted that the junction between the toxin leader peptide and the 5' end of the IL-1β sequence (Figure 1B) is formed by three amino acids (Thr-Arg-Gly) specified by the polylinker sequences preceding the *Bam*HI cloning site. The resulting plasmid, YEpsec1-hI1, should therefore direct the synthesis and secretion of a human IL-1β containing three additional amino acids at the amino terminus, unless the sequence Thr-Arg-Gly, which is itself a plausible signal sequence for leader peptide cleavage (von Heijne, 1983), was recognized as an endopeptidase cleavage site.

Expression

Plasmid stability and copy number. The *S. cerevisiae* strain S150-2B was transformed with YEpsec1 and YEpsec1-hI1 to produce the transformants Tsec1 and ThI1, respectively. Since strain S150-2B is *cir*$^+$, there is the possibility of recombination between the 2μm sequences present on YEpsec1 or YEpsec1-hI1 and the endogenous 2-μm plasmid or of a loss of the recombinant plasmid, when transformants are grown in the absence of selection (Erhart and Hollenberg, 1983). The stability of the URA$^+$ phenotype in the Tsec1 and ThI1 transformants was analysed by growing Tsec1 and ThI1 cells in non-selective medium for 20 generations and by plating of the same number of cells onto selective and non-selective media. The same number of colonies arose on both media, suggesting a stable phenotype.

A more quantitative analysis of the stability of the plasmid is shown in Figure 2A. Total yeast DNA prepared from strain AH22 grown in complete medium (lane b) and from a ThI1 transformant grown in synthetic medium lacking uracil (lane c) or in complete medium (lane d) was electrophoresed, transferred onto nitrocellulose filter and hybridized to labelled YEpsec1-hI1 DNA. In yeast transformants grown without selective pressure the YEpsec1-hI1 plasmid is stably maintained in an episomal state (lane d). The copy number of YEpsec1-hI1 is at least 2-fold greater than that of the 2-μm plasmid in the same transformant (lanes c and d). Furthermore, comparison between the intensity of hybridization of the 2-μm bands in AH22 DNA and that of the YEpsec1-hI1 bands in ThI1 DNA suggests that the YEpsec1-hI1 plasmid is present in high copy number (lanes b and d). This unusual phenotype is considered further in the Discussion. Figure 2B shows a gel electrophoresis of the same DNAs digested with the restriction enzymes *Pst*I and *Hpa*I that have unique sites within the vector and the 2-μm plasmid DNAs. The hybridization of ^{32}P-labelled YEpsec1-hI1DNA to total DNA from AH22 (lane b) and ThI1 cells (lanes c and d) digested with the *Pst*I restriction enzyme, shows a major band comigrating with that of the linearized YEpsec1-hI1 plasmid DNA (lane a). A similar result is obtained after digestion of the DNAs with the

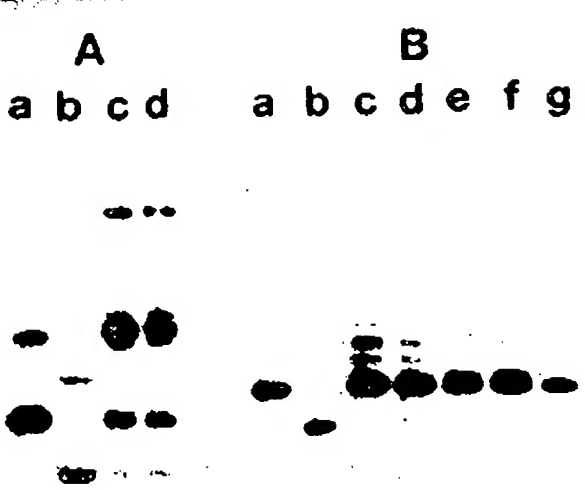

Fig. 2. Plasmid stability and copy number in yeast transformed with YEpsec1-hI1. Equal amounts (5 μg) of total DNA from AH22 and transformant ThI1 were resolved on an agarose gel, transferred to nitrocellulose and hybridized to ^{32}P-labelled YEpsec-hI1 DNA. Panel A Purified DNA of plasmid YEpsec1-hI1 (a). Total yeast DNA: from AH22 (b); from ThI1 grown in synthetic medium lacking uracil (c); or in complete medium (d). Panel B Purified DNA of plasmid YEpsec1-hI1 digested with *Pst*I (a) or *Hpa*I (g). Total yeast DNA: from AH22 digested with *Pst*I (b); from ThI1, grown in synthetic medium lacking uracil, digested with *Pst*I (c) or *Hpa*I (e); from ThI1, grown in complete medium, digested with *Pst*I (d) or *Hpa*I (f).

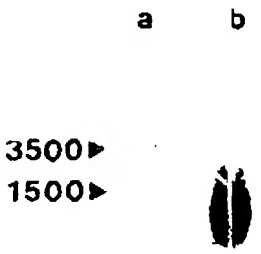

Fig 3. Transcription of the Il-1β sequence in yeast. An autoradiograph of a Northern blot of total RNA from transformant ThI1 grown in complete medium supplemented with 2% ethanol (a) or 2% galactose (b) is shown. The filter was hybridized with an Il-1β-specific probe. To the side the relative mobilities of *E. coli* rRNAs are indicated in nucleotides.

*Hpa*I restriction enzyme (lanes e, f and g). The fainter, fast migrating band of lanes b – f represent the 2-μm plasmid DNA present in all strains (compare lanes c – f with b). The slowly migrating bands present in lanes c, d and f are faint and most likely due to the anticipated recombination between YEpsec1-hI1 and the 2-μm plasmid by the *FLP*-mediated recombination system (Broach *et al.*, 1982). This result suggests that no major rearrangements of the YEpsec1-hI1 plasmid has occurred in the transformants.

Transcription of IL-1β sequences in YEpsec1-hI1. The transcription of the IL-1β cDNA sequences in transformant ThI1 cells was analysed by hybridization of total RNA extracted from ThI1 cells grown in media containing ethanol or galactose as a carbon source (Figure 3) to the pure ^{32}P-labelled IL-1β cDNA recombinant fragment. A transcript of ~1 kb could be detected only in the RNA prepared from ThI1 cells grown in galactose medium (Figure 3, lane b). This result shows that the IL-1β sequences present in YEpsec1-hI1 are transcribed under the control of the inducible hybrid promoter GAL-CYC and efficiently terminated, presumably at the *FLP* gene terminator.

Protein expression and secretion. The synthesis and secretion of IL-1β in ThI1 cells were assayed by SDS – polyacrylamide gel electrophoresis of whole cell extracts and of supernatants obtained after growth of ThI1 cells in galactose medium. As a control, the same analysis was extended to ThI1 cell cultures grown in ethanol medium and to Tsec1 cultures grown in medium containing either carbon source. The results are shown in Figure 4A. Coomassie Blue-stained protein profiles of cell extracts from ThI1 cells (lanes a and b) do not differ from those of cell extracts from Tsec cells (lanes e and f). Upon galactose induction of the GAL-CYC promoter, a new protein appears in the supernatant of ThI1 (lane c). This protein has an apparent mol. wt of 22 kd. By comparison of Coomassie Blue-stained protein profiles with calibrated standards, the amount of secreted 22-kd protein is 1 – 2 mg per litre of culture grown in complete galactose medium to stationary phase. Moreover, an immunoblotting analysis of the 22-kd protein in the supernatant and in the cell extract from ThI1 cells grown under these conditions has shown complete secretion of this protein into the culture medium (Figure 4B).

Assuming that the protein secreted by ThI1 is the recombinant Il-1β its mol. wt should be ~17 and not 22 kd (Auron *et al.*, 1984). Since the IL-1β sequence contains a potential glycosylation site (Asn-Cys-Thr) at the *N* terminus (Figure 1B), the discrepancy in mol. wt might be due to the presence of *N*-linked oligosaccharides. Digestion of the secreted protein with endoglycosidase H shows the disappearance of the 22-kd protein band and the appearance of a new band of ~17 kd (Figure 5, lane b) larger than 17 kd is possibly due to contamination of the endoglycosidase H enzyme by α-mannosidase that would release intermediate products of digestion. This result was confirmed by inhibiting glycosylation *in vivo* with tunicamycin. Supernatants from cultures of ThI1 grown in galactose medium containing tunicamycin at concentrations of the drug >0.5 μg/ml yield only the 17-kd protein (data not shown).

The 22-kd glycoprotein is recombinant human IL-1

Amino acid sequence of the secreted protein. The sequence of nine amino acid residues at the *N*-terminus of the putative IL-1β has been determined. The sequence (Ser-Leu-X-X-Thr-Leu-Arg-Asp-Ser) is in agreement with that specified by the 5' end of the IL-1β cDNA (Auron *et al.*, 1984; March *et al.*, 1985) cloned in YEpsec1 (Figure 1B). No additional amino acids encoded by

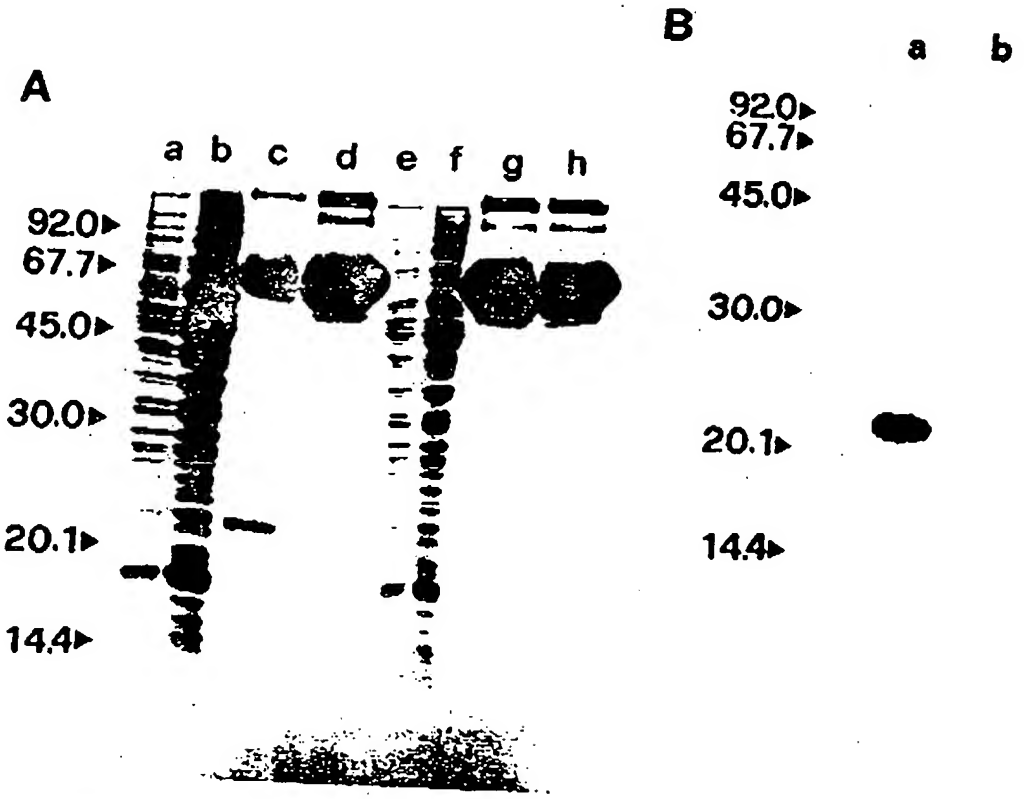

Fig. 4. Panel A Coomassie-stained SDS-polyacrylamide gel (SDS–PAGE) protein profiles. Lanes a–d Whole cell extracts (a and b) and culture supernatants (c and d) of ThI1 grown in complete medium supplemented with 2% galactose (a and c) or 2% ethanol (b and d). Lanes e–f Whole cell extracts (e and f) and culture supernatants (g and h) of Tsec1 grown in complete medium supplemented with 2% galactose (e and g) or 2% ethanol (f and g). The 67.7-kd band present in all culture supernatants is bovine serum albumin (BSA), used as a carrier for protein precipitation as described in Materials and methods. Panel B Western blot analysis of the 22-kd protein. Proteins from the supernatant (a) and from the cell extract (b) of ThI1 cells grown in complete medium supplemented with 2% galactose were separated by SDS–PAGE, transferred to nitrocellulose membrane and immunoblotted with a rabbit serum specific for the 22-kd protein. Mol. wt markers are shown in kd.

the polylinker sequence were found at the amino terminus of the protein, suggesting that cleavage of the leader peptide occurs between the last of the three amino acids specified by the vector polylinker sequences and the first amino acid of IL-1β.

Biological activity. The biological activity of the recombinant 22-kd protein was tested in a mouse thymocyte proliferation assay. After gel filtration on Sephacryl-S200 of 0.3 ml of ThI1 cell culture supernatant, aliquots of the eluted fractions were added to the thymocyte cultures and the [^{3}H]dThd incorporation into proliferating thymocytes was measured. Figure 6 shows that IL-1 activity can be recovered from fractions falling into the right range of mol. wt, with a maximal stimulation of 7-fold above background. This result shows that the 22-kd protein secreted by *S. cerevisiae* has properties similar to those of natural IL-1β.

Discussion

The construction of the pEMBLyex2 plasmid and its secretion derivative YEpsec1 was designed to introduce novel features into an otherwise typical yeast–bacterial shuttle vector (Parent *et al.*, 1985). The presence of the f1 phage origin of replication allows purification of the plasmid in a single-stranded form. The pEMBLyex2 plasmid also contains the multiple site polylinker of the pEMBL 18$^+$ vector (Dente *et al.*, 1985). Seven unique

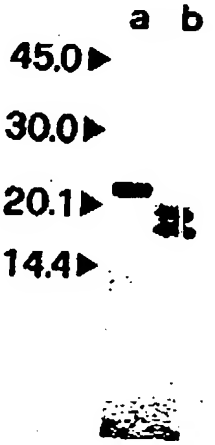

Fig. 5. Endoglycosidase H treatment of the 22-kd protein. Coomassie-stained SDS–PAGE profile of the 22-kd protein before (a) and after (b) treatment with endoglycosidase H. The protein was purified on a 5 × 90 cm Ultrogel AcA54 column equilibrated with phosphate buffer saline (PBS), incubated with endoglycosidase H (a gift from C.Ceccarini) in 20 mM citric acid, 40 mM Na_2HPO_4 at 37°C for 16 h and precipitated with 10% TCA. Mol. wt markers, expressed in kd, are shown.

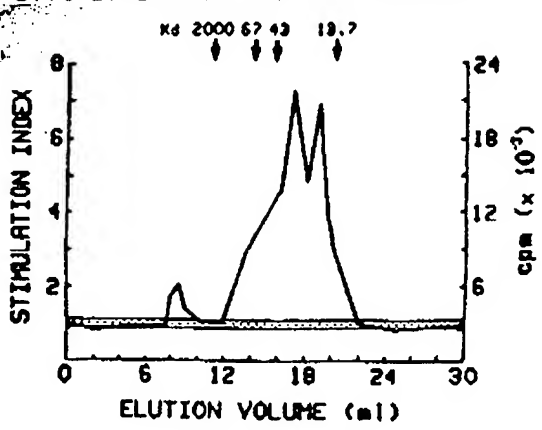

Fig. 6. Biological activity of recombinant IL-1β. The graph shows the stimulation of mouse thymocyte proliferation by gel filtration fractions of the supernatant from a culture of Th11 grown in galactose medium. Abscissa: elution volume (ml) from the Sephacryl-S200 column. Ordinate: incorporation of [^{3}H]thymidine by mouse thymocytes. Background incorporation stimulated by phytohemagglutinin (PHA) alone is shown (dotted area). Mol. wt standards are indicated by the arrows. Each point is the mean of triplicate determinations with SEM < 10%.

recognition sites for restriction endonucleases (*Sst*I, *Sma*I, *Xma*I, *Bam*HI, *Sal*I, *Pst*I, *Hin*dIII) are provided by the polylinker sequence in pEMBLyex2 to allow insertion of genes downstream of the hybrid promoter GAL–CYC and upstream of a yeast transcription terminator. Both these elements are required for efficient expression in yeast (Hitzeman *et al.*, 1983). Moreover, in YEpsec1 a synthetic sequence was introduced downstream of the *Sst*I site which reproduces the hypothetical leader peptide sequence of the killer toxin *K. lactis*. This toxin is encoded by the ORF2 of the *K. lactis* K1 (or pGK11) killer plasmid (Stark *et al.*, 1984). The *N*-terminal region of the toxin deduced from its nucleotide sequence is composed of a hydrophylic carboxyterminal end followed by a hydrophobic core and the plausible endopeptidase cleavage signal Val-Gln-Gly (Perlman and Halvorson, 1983). Although we have not proved that this sequence represents the leader peptide of the killer toxin, we have shown that it is effective in directing the secretion of a heterologous mammalian protein. The absence of the recombinant IL-1β in the whole cell extract of the transformed yeast cultures shows that the majority of the protein is secreted and that the YEpsec1 plasmid is an efficient secretion vector for the production of a foreign protein.

An additional feature of pEMBLyex2 and YEpsec1 is the presence of both *URA3* and *leu2-d* as yeast auxotrophic selectable markers. *Leu2-d* was originally isolated on pJBD219 (Beggs, 1978) and is a poorly expressed allele of *LEU2* which appears to increase the stability and copy number of 2-μm plasmid derivatives under non-selective conditions (Futcher and Cox, 1984). Under leucine deprivation, copy number of *leu2-d* plasmids rises sufficiently high to cure the endogenous 2-μm plasmid (Erhart and Hollenberg, 1983). However *leu2-d* is a poor selectable marker for Li^+-mediated transformation (data not shown) so *URA3* was also included.

Indeed the YEpsec1 plasmid, which relies for its maintenance in yeast on the 2-μm origin of replication derived from pJBD219, was found to be highly stable when introduced into a *S. cerevisiae* strain carrying the natural 2-μm plasmid. The copy number of the plasmid appeared to be equally high in yeast transformants grown under selective (uracil) or non-selective conditions. Thus, the *ORI–STB cis*-acting sequences (Kikuchi, 1983) present in YEpsec1 are sufficient for stable propagation at high copy number of the plasmid even in the absence of selection, provided that the functions for replication and stability are supplied *in trans* by the endogenous 2-μm plasmid.

As shown by the amino acid sequence analysis of secreted IL-1β, the site of cleavage of the hybrid protein occurs at the junction between the polylinker sequence and the first amino acid specified by the IL-1β cDNA sequence, which is three amino acids downstream of the presumptive cleavage site of the killer toxin. According to the set of rules formulated by von Heijne (1983) the presence of residues Thr and Gly at positions −3 and −4, respectively, in a cleavage signal sequence results in a higher 'processing probability' of the site as compared with the presence of the Val and Phe residues at the same positions. Our results confirm von Heijne's predictions, and show that the addition of the polylinker sequence produces a new and efficient endopeptidase cleavage site in *S. cerevisiae*.

Materials and methods

Strains and media

The following *S. cerevisiae* strains were used: S150-2B (*leu2-3 leu2-112 ura3-52 trp1-289 his3-Δ1 cir*$^+$) and AH22 (*leu2-3 leu2-112 his4-519 can1 cir*$^+$). Strain S150-2B was transformed by the LiCl method (Rothstein, 1985). Yeast cells were grown in synthetic medium containing 2% carbon source and 0.67% yeast nitrogen base (Difco) supplemented with the required amino acids (50 μg/ml) or in complete medium containing 2% carbon source, 1% yeast extract, 2% peptone.

The *E. coli* strain 71-18 (*lac pro*− *supE thi*$^-$/*F'-lac*I^q *lacZ*Δ *M15 proB*$^+$ *A*$^+$) or HB101 (*F*$^-$ *hsdS20 recA13 ara-14 proA2 lacY1 galK2 rpsL20 xyl-5 mtl-1 supE44*) were used as recipient cells for plasmid constructions. Transformation of *E. coli* cells and analysis of recombinant plasmids was carried out as described by Maniatis *et al.* (1982).

Plasmid construction

The steps followed for constructing pEMBLyex2 are outlined in Figure 1A. Plasmid YEpsec1 was obtained by inserting a 51-bp-long synthetic oligonucleotide (Figure 1B) between the *Sst*I and *Kpn*I restriction sites of the pEMBLyex2 plasmid polylinker. The oligonucleotide was synthesized on an Applied Biosystems 380A DNA synthesizer. The correct insertion and sequence of the oligonucleotide into plasmid YEpsec1 was verified by the dideoxy method as described by Sanger *et al.* (1977).

Plasmid YEpsec1-hI1 was constructed by inserting into the *Bam*HI site of the YEpsec1 vector a *Sau*3A fragment containing a 600-bp-long segment of human IL-1β cDNA (G.Bensi, unpublished data).

Nucleic acids purification and analysis

Total yeast DNA was isolated as described by Davis *et al.* (1980). After digestion with restriction endonucleases, the DNA was fractionated on 1% agarose gel and transferred to nitrocellulose filter (Southern, 1975). The filter was hybridized to YEpsec1-hI1 DNA labelled by nick translation. Total yeast RNA was prepared as described by Rubin (1975) and purified from plasmid DNA by incubation with 60 ng/ml DNase I in 10 mM $CaCl_2$, 1 × DNase buffer (20 mM Tris–HCl, pH 7.4, 10 mM $MgCl_2$) in the presence of 30 μg/ml proteinase K. After fractionation on a formaldehyde agarose gel, the RNA was transferred to nitrocellulose filter (Maniatis *et al.*, 1982) and probed with the IL-1β DNA fragment used for cloning. Prior to labelling, this DNA fragment was purified on a low melting point agarose gel (Miles) as described by Crouse *et al.* (1983).

Protein purification and analysis

Whole-cell extracts were prepared from yeast cultures by vortexing cell pellets with glass beads for 2 min in the cold. After disruption, the cell suspensions were clarified by centrifugation at 10 000 r.p.m. for 15 min and diluted in sample buffer to 2% SDS, 10% glycerol, 5% mercaptoethanol, 62.5 mM Tris–HCl, pH 6.8.

Culture supernatants were filtered through Millipore filters (0.45 μm) before undergoing protein analysis. Proteins were precipitated from supernatants in the presence of 100 μg/ml of BSA with 10% TCA. Samples were incubated at −20°C for 20 min, centrifuged for 10 min in an Eppendorf microfuge and the protein pellet resuspended in SDS sample buffer. Proteins were analysed by SDS–polyacrylamide gel electrophoresis as described by Laemmli (1970).

Protein samples subjected to SDS–polyacrylamide gel electrophoresis were transferred to nitrocellulose as described by Towbin *et al.* (1979). The nitrocellulose membrane was incubated for 2 h in a buffer containing PBS, 3% BSA and 0.1% Triton. Polyclonal antiserum, raised in rabbit against the 22-kd protein (a gift from J.Telford), was then added at a 1:100 dilution in the same buffer. The blot was developed with horseradish peroxidase conjugate (1:1000 dilution, Cappel) using 4-chloro-1-naphthol and H_2O_2 as substrates.

For *N*-terminal sequence determination, the protein was alkylated by treatment with 20 mM iodoacetic acid, subjected to preparative gel electrophoresis and electroeluted as described by Hunkapiller *et al.* (1983). The sequence at the *N*-terminal end was obtained by the Edman degradation method using a gas-phase protein sequencer (Hewick *et al.*, 1981).

Thymocyte proliferation assay

The culture supernatant was concentrated 10-fold by ultrafiltration and applied to a 30 × 1 cm Sephacryl-S200 column equilibrated with 0.15 M NaCl. The flow rate was 1 ml/min and 0.5 ml fractions were collected and assayed for IL-1 activity in the murine thymocyte proliferation assay as described by Gery *et al.* (1981). Briefly, thymocytes from 4- to 8-week-old C3H/HeJ mice (6×10^5 cells/well of Cluster[96] plates, Costar) were exposed for 72 h to a 1/4 dilution of each column fraction in the presence of 1.5 μg/ml purified phytohemagglutinin (PHA, Wellcome) in 0.2 ml RPMI 1640 (Gibco) supplemented with 5% fetal bovine serum (FBS, Hy-clone, Sterile Systems), 50 μg/ml gentamycin sulphate (Sigma), 25 mM Hepes buffer, 2 mM L-glutamine and 1.25×10^{-5} M 2-mercaptoethanol. Cultures were then pulsed with 1 μCi/well [^{3}H]dThd for 16–18 h, harvested on glass fiber filters and assayed for incorporation of radioactivity by liquid scintillation spectrometry. Proliferation was measured as c.p.m. ± SEM of triplicate cultures and expressed as stimulation index, i.e. the ratio between experimental (PHA + fraction) and control (PHA only) groups.

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